Effect of Infant Formula on *Streptococcus Mutans* Biofilm Formation

Laura M Hinds[*] / Elizabeth A S Moser[**] / George Eckert[***]/ Richard L Gregory[****]

Objective: *This study investigated the effect that infant formula had on biofilm growth of Streptococcus mutans. Specifically, it compared biofilm growth in media containing lactose-based and sucrose-based formulas. It also analyzed biofilm formation with formulas of varying iron content. Biofilm growth was tested with the specific infant formula components sucrose, lactose, and ferric chloride. The study was designed to determine if these types of infant formulas and components affected S. mutans biofilm formation differently.* ***Study design***: *A 24-hour culture of S. mutans was treated with various concentrations of infant formula diluted in bacteriological media. To test for biofilm formation, S. mutans was cultured with and without the infant formula and formula components. The biofilms were washed, fixed, and stained with crystal violet. The absorbance was measured to evaluate biofilm growth and total absorbance.* ***Results***: *Sucrose-based formulas provided significant increases in biofilm growth when compared to lactose-based formulas at two dilutions (1:5, 1:20). Similac Sensitive RS (sucrose-based) at most dilutions provided the most significant increase in biofilm growth when compared to the control. Sucrose tested as an individual component provided more of a significant increase on biofilm growth than lactose or iron when compared to the control. A low iron formula provided a significant increase in biofilm growth at one dilution (1:5) when compared to formula containing a normal iron content. There was no significant difference in biofilm growth when comparing high iron formula to normal iron formula or low iron formula. There was no significant difference when comparing Similac PM 60/40 (low iron formula) to Similac PM 60/40 with additional ferric chloride.* ***Conclusion*** *:The results of this study demonstrated that sucrose-based formula provided more of a significant increase in biofilm growth compared to lactose-based formula. Sucrose alone provided a significant increase of biofilm growth at more dilutions when compared to the control than lactose and iron. The amount of iron in formula had a significant effect on biofilm formation only when comparing low iron formula to normal iron formula at the highest concentration (1:5). There was no significant difference in biofilm growth when iron was added to the low iron formula. The information obtained expands current knowledge regarding the influence of infant formula on the primary dentition and reinforces the importance of oral hygiene habits once the first tooth erupts.*

Key Words: infant formula, sucrose, lactose, ferric chloride, early childhood caries (ECC), S. mutans

INTRODUCTION

Infant formula is a safe source of food for non-breastfed infants up to six months old and continues as an important food source for infants through their first year.[1] Many parents make the decision not to breastfeed and rely on infant formula for nutrition. According to a recent study, 83% of those surveyed initiated breast-feeding: 50% of those were still breastfeeding at 6 months and only 24% continued at 12 months. In the survey, 52% reported their infants received formula in the hospital after birth.[2] According to the CDC, although the majority of infants in the United States begin breastfeeding, most receive formula by six months, either instead of or as a supplement to breast milk.[3]

As reported by the World Health Organization,[4] the quantity of nutrients in formula are adjusted to make them comparable to breast milk. Infant formula is altered cow's milk with the addition of fluoride through water.[5] Formula differs from breast milk in that

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anti-infective and bioactive factors cannot be added, and the fat and protein content of formula cannot be altered.[4] A study completed by Holgerson *et al* [6] found that the oral flora differed in breastfed infants compared to formula fed infants. Lactobacilli were detected in the saliva of breastfed infants but not detected in formula fed infants. Lactobacilli inhibit the growth of cariogenic oral pathogens including *Streptococcus mutans*. This indicates that breastfed infants have a potentially healthier oral flora.[6]

According to the CDC, dental caries is the most prevalent infectious disease for children in the United States.[7] Severe early childhood caries is defined as any sign of smooth surface caries in a child under the age of three.[8] Severe early childhood caries or "baby bottle" tooth decay is correlated with infants who sleep with a bottle. According to a study by Kaste and Gift,[9] nearly 20% of children between the ages of six months and five years have been put to bed with a bottle filled with something other than water.[9] This behavior leads to the development of early childhood caries. This type of decay is strongly associated with a high percentage of *S. mutans* and normally is seen on the maxillary anterior deciduous dentition.[10] The caries potential is increased because the amount of saliva contacting the teeth is decreased and blocked by the nipple on the bottle.[11]

S. mutans is the main bacterial organism that metabolizes sugars, producing acid which demineralizes tooth structure and causes dental caries.[12] Those with a higher caries rate have a higher percentage of *S. mutans* compared to a lower percentage in caries free individuals. *S. mutans* is an important cariogenic bacteria in most dental biofilms.[13] A biofilm is an aggregation of microorganisms that attach to each other or a surface, such as a tooth, and enclose themselves in a self-produced extracellular polymeric substance (EPS). Carbohydrates cause biochemical and physiologic changes to biofilm and enhance the cariogenic properties.[13]

The carbohydrate content of infant formula is either sucrose or lactose based. Sucrose is considered the most cariogenic dietary carbohydrate because it is fermentable by oral bacteria. This process causes the pH of the environment to decrease thus influencing microflora in the oral cavity to be more cariogenic.[14] Sucrose also acts as a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides in dental plaque. Extracellular polysaccharides promote bacterial adherence to the tooth surface and enforces the structure of the biofilm.[14] Biofilms formed in the presence of sucrose had lower pH levels, a higher *S. mutans* count, and an enhanced cariogenic potential compared to those without sucrose.[14] Lactose is a disaccharide that is derived from galactose and glucose and it is commonly found in dairy rich diets. Lactose is rapidly fermented by *S. mutans* in the oral cavity.[15] In a study completed by Campbell and Zinner,[16] the comparative effect of sugary diets including sucrose, fructose, glucose, or lactose on hamster dental caries was examined. Sucrose was the most cariogenic sugar tested and it demonstrated the most rapidly progressive carious process. Over time, fructose, lactose, and glucose also caused dental destruction but not to the same extent as sucrose.[16]

Many studies have been completed examining the cariogenic potential of infant formula, cow's milk, and breast milk. According to a study by Peres *et al*[17] the results indicated that infant formula had cariogenic properties and was as cariogenic as sucrose; however, the addition of fluoride (10 ppm) reduced the cariogenic potential of the formula. Results also demonstrated that breast milk had a small cariogenic potential and cow's milk was negligible in the caries forming process.[17] In addition, a study by Bowen and Lawrence[18] concluded that breast milk has some potential to promote caries; however, cow's milk was found not to promote caries. Similarly, a study by Bowen et al. [11] stated that infant formulas had higher carbohydrate variability and greater potential to promote cariogenic properties. Plain milk was the least cariogenic.[11] In an in vitro caries study, Prabhakar *et al* [19] found that sweetened cow's milk supported maximal bacterial growth compared to plain cow's milk. Human breast milk supported the least amount of bacterial growth. The predominant sugar in plain cow's milk and human breast milk was lactose as compared to sucrose in the sweetened milk.[19] Lactose does not lower pH values as drastically as sucrose. The study concluded that the addition of an external carbohydrate source to milk, such as sucrose, increases the cariogenic potential and the extent of caries progression into dentin.[19]

Formulas contain iron to prevent iron deficiency anemia in the newborn.[20] However, iron has numerous important roles in bacterial cells that influence cell growth and composition. Iron deficiency can inhibit growth and decrease RNA and DNA synthesis of bacteria.[21] Bacteria rely on nutrition from the host and the environment to survive. Iron is the nutritionally limiting factor for bacterial multiplication in serum, saliva, tears and milk.[21] When bacteria invade mammals, the animal, through the action of factors such as lactoferrin or transferrin, limit the amount of available iron to prevent growth and spread of infection.[21] On the contrary, a study conducted by Ribeiro *et al* [22] concluded that iron treatment reduced the number of bacteria formed in an *S. mutans* biofilm and the increased iron reduced enamel demineralization. After biofilms were exposed to different amounts of iron, it was found that iron ions interfered with the enzymes that allow bacteria to adhere in dental biofilms.[22] Similarly, in an *in situ* study by Marthinhon,[23] iron reduced the amount of demineralization of bovine enamel submitted to a cariogenic environment. Also, the ionic composition of dental biofilm was altered with an increased amount of phosphorous and iron.

MATERIALS AND METHOD

S. mutans strain UA159 (ATTC strain 700610) was used in the present study due to its completely sequenced genome. The strain was stored at -80°C in tryptic soy broth without dextrose (TSB w/o dextrose, *Acumedia, Baltimore, MA*) with 20% glycerol before use. Mitis Salivarius Sucrose Bacitracin (MSSB, Anaerobe Systems, Morgan Hill, CA) agar plates were used to initially grow the strains. Unless otherwise stated, TSB without dextrose was used and the growth conditions were 95% air and 5% CO_2 at 37°C.[15] The eleven (six lactose-based and five sucrose-based) infant formulas (Table 1) were purchased from a local grocery store and were used within three months of purchase.

Biofilm Formation

To determine biofilm formation, an overnight *S. mutans* culture (10^6 CFU/ml) in TSB without dextrose was treated for 24 hours with various concentrations of the eleven infant formulas diluted in TSB. A preliminary test was conducted to determine the best formula concentrations to use. Each formula was tested at the following dilutions: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1280.

Table 1. Composition of infant formulas used in the study

Infant Formula	Carbohydrate Source (g) Replete Concen.	Protein Source (g) Nutrient Composition Per 100 cal (5 fl oz)	Amount of Iron (mg) Replete Concen.	Fat Source (g)
Similac PM 60/40 Powder Low Iron Infant Formula	Lactose (100%) 10.20 g 69.00 mg/ml Lactose	Nonfat Cow's Milk, Whey Protein, Sodium Caseinate 2.20 g	0.70 mg 0.0047 mg/ml	High Oleic Safflower, Soy and Coconut Oil 5.60 g
Similac Advance (DHA and AA Formula)	Lactose, Galacto-oligosaccharides (94:6) 10.71 g 68.06 mg/ml Lactose	Nonfat Cow's Milk, Whey protein 2.07 g	1.80 mg 0.0122 mg/ml	Palm Olein, Coconut and Soy Oil, High Oleic Safflower 5.60 g
Similac Go and Grow Milk Based (Toddler Formula)	Lactose, Galacto-oligosaccharides (94:6) 10.20 g 62.32 mg/ml Lactose	Nonfat Cow's Milk, Whey Protein 3.00 g	2.00 mg 0.0135 mg/ml	High Oleic Safflower, Soy and Coconut Oil 5.40 g
Enfamil Premium Infant	Lactose, Galacto-oligosaccharides (92:8) 11.20 g 69.66 mg/ml Lactose	Nonfat Cow's Milk, Whey Protein 2.10 g	1.80 mg 0.0122 mg/ml	Palm Olein, Coconut, Soy and High Oleic Sunflower Oil 5.30 g
Enfamil Premium Newborn	Lactose, Galacto-oligosaccharides (93:7) 11.20 g 70.33 mg/ml Lactose	Nonfat Cow's milk, Whey Protein 2.10 g	1.80 mg 0.0122 mg/ml	Palm Olein, Soy, Coconut, and High Oleic Sunflower Oil 5.30 g
Enfamil EnfaCare	Lactose, Corn Syrup Solids (65:35) 10.40 g 45.99 mg/ml Lactose	Nonfat Cow's Milk, Whey Protein 5.30 g	1.80 mg 0.0122 mg/ml	High Oleic Safflower, Soy and Coconut Oil, Medium Chain Triglycerides 2.80 g
Similac Sensitive RS (Anti-Reflex Formula)	Corn Maltodextrin, Sucrose, Galacto-oligosaccharides (53:44:3) 11.10 g 33.04 mg/ml Sucrose	Milk Protein Isolate 2.14 g	1.80 mg 0.0122 mg/ml	High Oleic Safflower, Soy and Coconut Oil 5.40 g
Similac Alimentum (Hypoallergenic/ Protein Hydrolysate)	Sucrose, Modified Tapioca Starch (70:30) 10.20 g 48.30 mg/ml Sucrose	Extensively Hydrolyzed/ Casein hydrolysate, L-Systine, L-Tyrosine, L-Tryptophan 5.54 g	1.80 mg 0.0122 mg/ml	High Oleic Safflower, Medium Chain Triglycerides, Soy Oil 2.75 g
Similac Isomil (Soy Formula)	Corn syrup solids, Sucrose, Fructo-oligosaccharides (78:19:3) 10.40 g 13.24 mg/ml Sucrose	Soy Protein and L-Methionine 2.45 g	1.80 mg 0.0122 mg/ml	High Oleic Safflower, Coconut and Soy Oils 5.46 g
Similac For Spit-up (Anti Spit-Up)	Corn syrup, Rice starch, Sucrose, Galacto-oligosaccharides (48:30:19:3) 10.96 g 13.36 mg/ml Sucrose	Milk Protein Isolate 2.14 g	1.80 mg 0.0122 mg/ml	High Oleic Safflower, Soy Oil, Coconut Oil 5.40 g
Similac Go and Grow Soy Based (Soy Formula)	Corn Syrup Solids, Sucrose, Fructo-oligosaccharides (77:20:3) 10.30 g 13.92 mg/ml Sucrose	Soy Protein Isolate, L-Methionine 2.80 g	2.00 mg 0.0135 mg/ml	High Oleic Safflower, Soy Oil, Coconut Oil 2.50 g

Sucrose, lactose, and ferric chloride were tested at concentrations representative of the average infant formula concentration along with two fold dilutions above and below the average. The average infant formula concentrations of sucrose (24.37 mg/ml), lactose (64.23 mg/ml), and ferric chloride (0.0125 mg/ml) were calculated based on the formula used. Ferric chloride (2 mg/ml) was added to the 1:5 dilution of Similac PM 60/40 and the mixture diluted to 1:1280. The formulas were diluted with TSB without dextrose (190 µl total volume) and incubated with 10 µl of an overnight culture of *S. mutans* for 24 hours in sterile 96-well microtiter plates. The total absorbance of the wells indicating the relative amount of biofilm and planktonic cells were measured at 595 nm. Biofilm was washed twice with saline, fixed with 10% formaldehyde (Sigma) for 30 minutes, washed twice again with saline, and stained with 0.5% crystal violet for 30 minutes.[24] After washing the biofilm three times with saline, crystal violet was extracted from the biofilm cells by 200 µl of 2-propanol (Fisher Scientific, Co., Fair Lawn, NJ) for 1 hour. The extract was diluted 1:5 with 2-propanol and read at 490 nm with 2-propanol used as a blank control.[24]

Statistical analyses

Each experiment for the different types of formula was repeated individually three times. Summary statistics (mean, standard deviation, standard error, range) were calculated for each dilution for biofilm mass. Comparisons were made between each formula dilution and the control, between the low iron, normal iron, and high iron formulas, between the five sucrose-based and six lactose-based formulas, and between ferric chloride added to the low iron infant formula and the low iron formula without additional ferric chloride. ANOVA and Fisher's Protected Least Significant Differences multiple comparison tests were performed to compare the groups.[24] A 5% significance level was used for all tests. Based on the results of previous studies, the expected within-group standard deviations for biofilm mass was 0.03. Therefore this study had 80% power to detect biofilm mass of 0.3 between any two groups.

RESULTS

When comparing biofilm growth to the TSB without dextrose control, six (four of the sucrose-based and two of the lactose-based formula) of the eleven formulas tested had a significant increase in biofilm growth in multiple dilutions. Overall, biofilm growth tended to decrease as the sucrose-based formula concentrations decreased (Figure 1). For the majority of the dilutions, biofilm growth was greater when compared to the control and had a downward trend as the dilution increased. Four of the five sucrose-based formulas had statistically significant increases ($p<0.05$) in biofilm growth at multiple dilutions. Similac Sensitive RS had the most dilutions (seven; 1:5 - 1:320) with significant increases in biofilm growth. Similac Isomil had significant increases in biofilm growth at four dilutions (1:5 - 1:40). Similac for Spit Up and Similac Go and Grow Soy had significant increases in biofilm growth at three dilutions (1:20, 1:40, 1:80 and 1:5, 1:10, 1:20, respectively). Similac Alimentum had no significant changes in biofilm growth at any dilution (Figure 1).

The sucrose-based formulas were analyzed for statistically significant differences ($p<0.05$) in total growth. Overall, as sucrose formulas increased in dilution the total growth decreased (Figure 1). Similac Isomil, Similac Alimentum, and Similac for Spit Up had the most significant increases in total growth at all nine dilutions (1:5 - 1:1280). Similac Sensitive RS had significant increases at eight dilutions (1:5 - 1:640). Similac Go and Grow Soy had significant increases at six dilutions (1:5 - 1:160).

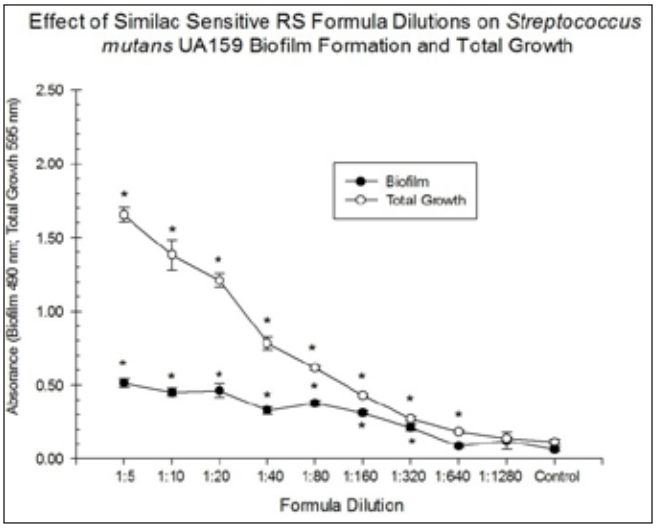
As the lactose-based formula concentrations decreased, biofilm growth tended to decrease (Figure 2). For the majority of dilutions, biofilm growth was greater when compared to the control and had a steady downward trend as the dilution increased. Two of the six lactose-based formulas had biofilm growth that was statistically significant ($p<0.05$) at specific dilutions. Similac Go and Grow had significant increases in biofilm growth at five dilutions (1:5 thru 1:80). Similac PM 60/40 had significant increases in biofilm growth at four dilutions (1:5, 1:10, 1:20, 1:80). Enfamil EnfaCare, Enfamil Premium Infant, Enfamil Premium Newborn, and Similac Advance had no significant differences in biofilm growth when each dilution was compared to the control (Figure 2).

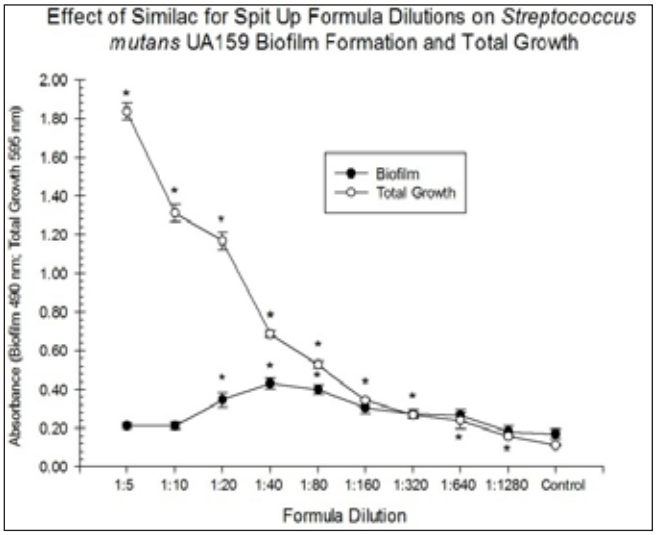
The lactose-based formulas were analyzed for statistically significant differences ($p<0.05$) in total growth. Overall, for the lactose-based formulas, as the dilution increased the total growth decreased. Five of the six lactose-based formulas had significant increases in total growth at all nine dilutions when compared to the total growth of the control (Figure 2). These five formulas included: Enfamil EnfaCare, Enfamil Premium Infant, Enfamil Premium Newborn, Similac Go and Grow, and Similac PM 60/40. Similac Advance had significant increases at five dilutions (1:5 thru 1:80).

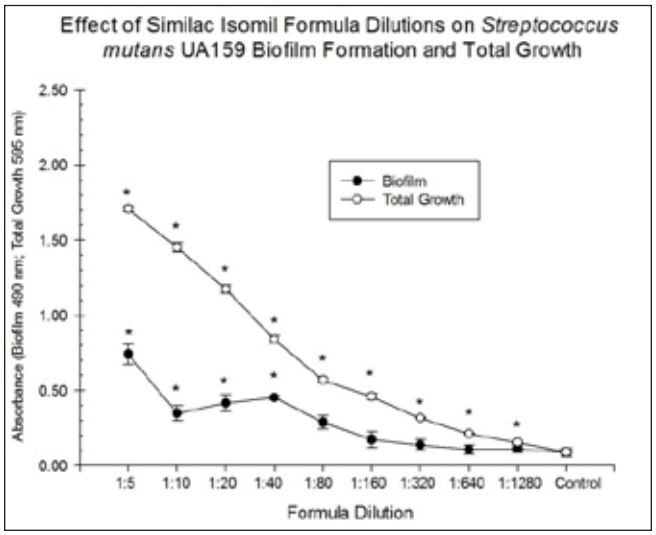
Sucrose, tested as an individual component and diluted in TSB, had statistically significant increases ($p<0.05$) in biofilm formation and total growth when compared to the control at eight concentrations (192, 96, 48, 24, 12, 6, 3, and 1.5 mg/ml). Biofilm growth increased and peaked at 12 mg/ml and then decreased with decreasing concentrations of sucrose (Figure 3). Lactose had no significant changes in biofilm growth at any of the concentrations. Biofilm growth tended to increase compared to the control until it peaked at 64 mg/ml and growth tended to decrease with decreasing concentrations of lactose. Lactose had statistically significant changes in total growth at eight concentrations (1024, 512, 256, 128, 64, 32, 16, 8 mg/ml). $FeCl_3$ had significant changes in biofilm growth at five concentrations. Biofilm growth was significantly increased at two concentrations of $FeCl_3$ (0.05 and 0.025 mg/ml) and was significantly inhibited at three concentrations of $FeCl_3$ (1, 0.0015625 and 0.00078125 mg/ml). Biofilm growth increased and peaked at 0.05 mg/ml ferric chloride and decreased with decreasing concentrations. $FeCl_3$ had significant changes in total growth at eight concentrations (0.0015625 – 1.0 mg/ml). For each component, total growth tended to increase or decrease in relation to the corresponding biofilm growth.

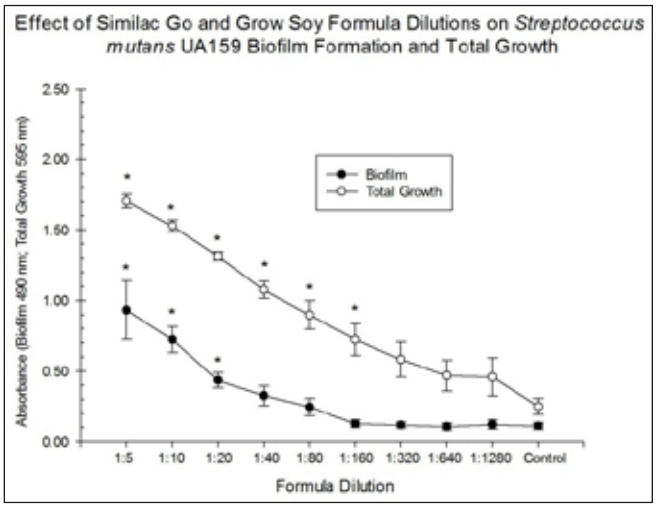
Sucrose formulas had statistically significant increases ($p<0.05$) in biofilm formation when compared to lactose formulas at two dilutions (1:5 and 1:20). The low iron content formula had a significant increase in biofilm growth when compared to the normal iron content formulas at one dilution (1:5). There were no significant differences in biofilm formation when comparing the dilutions of formula with high iron content to dilutions of formula with low and normal iron content. There were no significant differences in biofilm growth at any dilution when comparing Similac PM 60/40 + $FeCl_3$ to Similac PM 60/40 (Figure 4). $FeCl_3$ + Similac PM 60/40 had significant increases in biofilm growth at six dilutions (1:5 - 1:160) compared to the control. $FeCl_3$ + Similac PM 60/40 had significant increases in total growth at nine dilutions (1:5 - 1:1280) compared to the control.

Figure 1. Effect of 5 sucrose-based infant formulas on *S. mutans* biofilm formation and total growth compared to the TSB control without dextrose. Asterisks indicate statistically significant differences ($p<0.05$) compared to control values without formula.









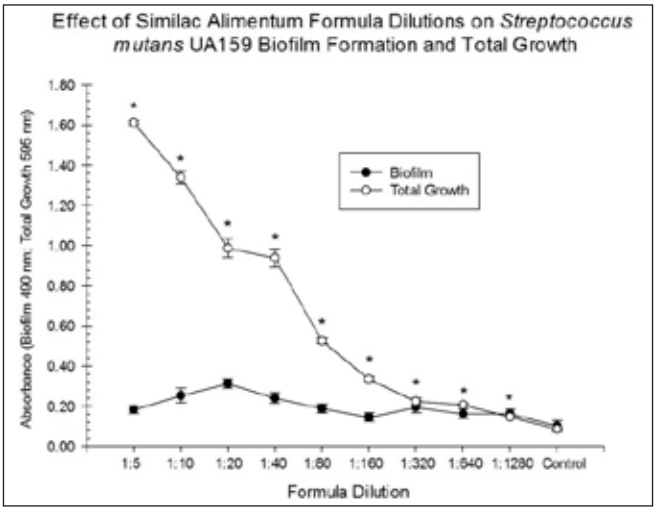
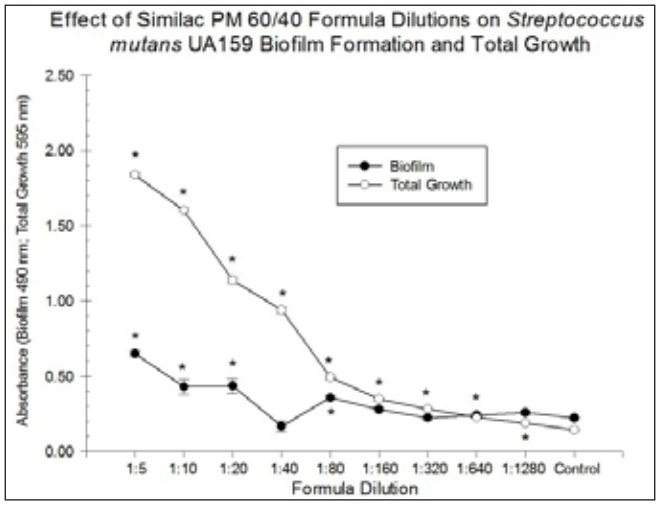
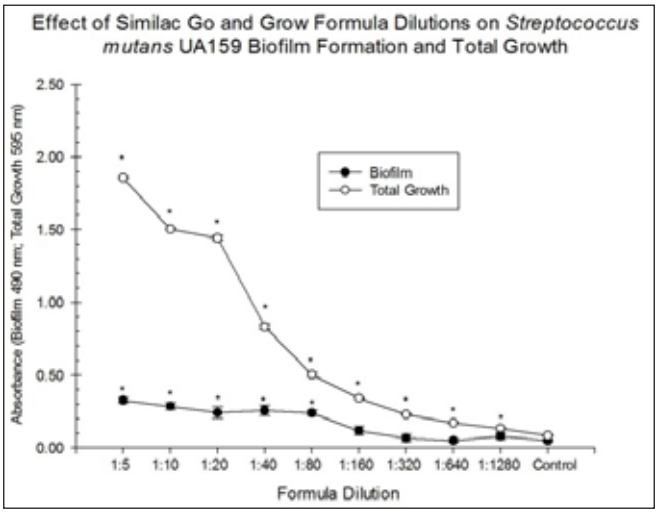
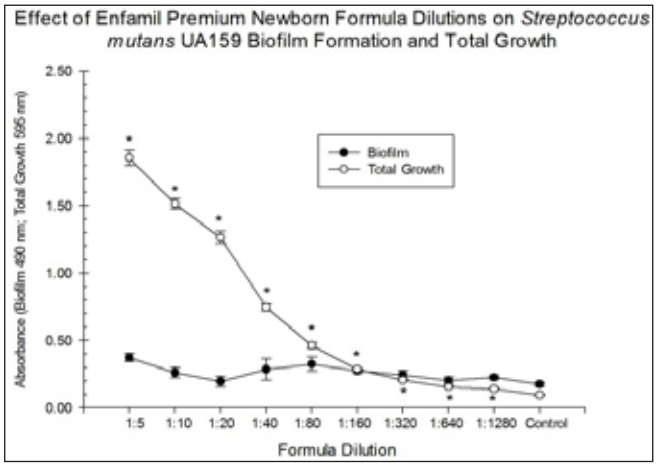
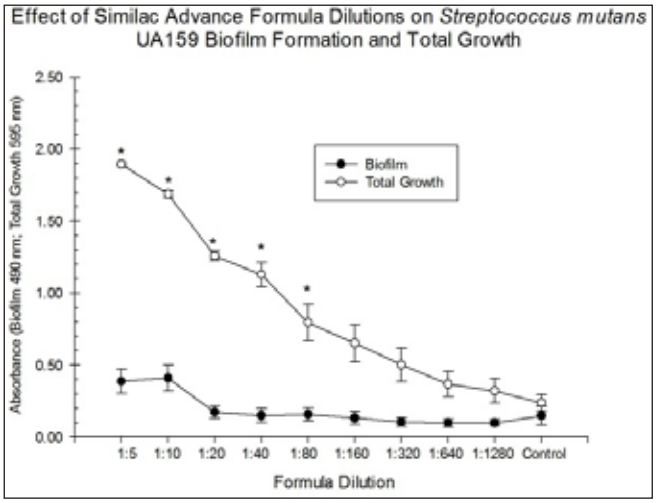


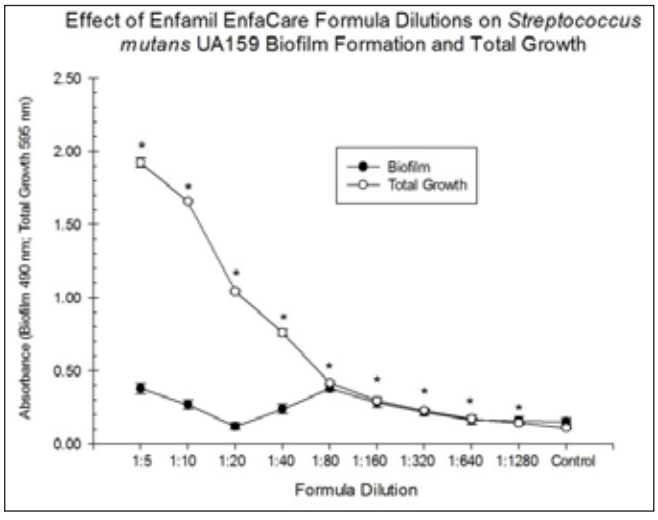
Figure 2. Effect of 6 lactose-based infant formulas on *S. mutans* biofilm formation and total growth compared to the TSB control without dextrose. Asterisks indicate statistically significant differences ($p<0.05$) compared to control values without formula.











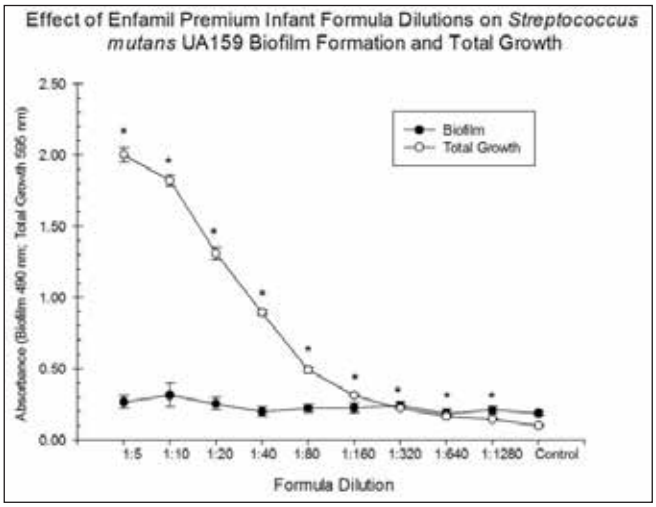
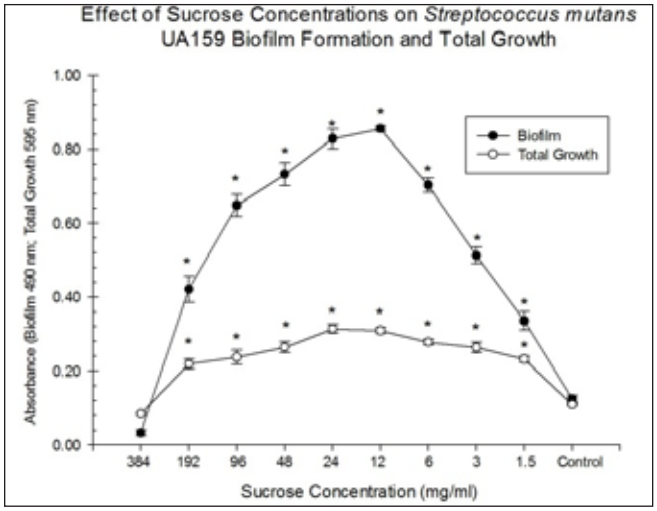
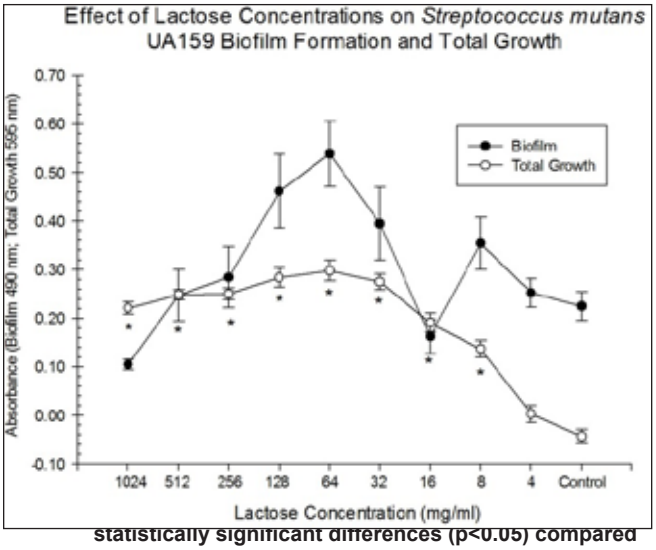


Figure 3. Effect of different concentrations of major infant formula components (sucrose, lactose, and ferric chloride) on *S. mutans* biofilm formation and total growth compared to the TSB control without dextrose. Asterisks indicate statistically significant differences (p<0.05) compared to control values.





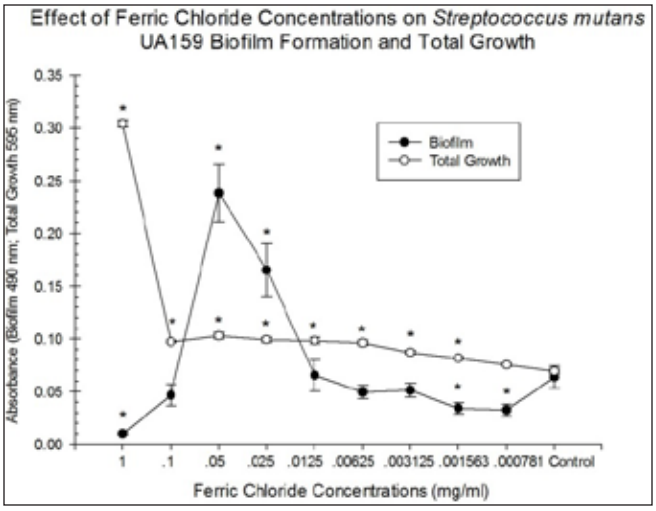
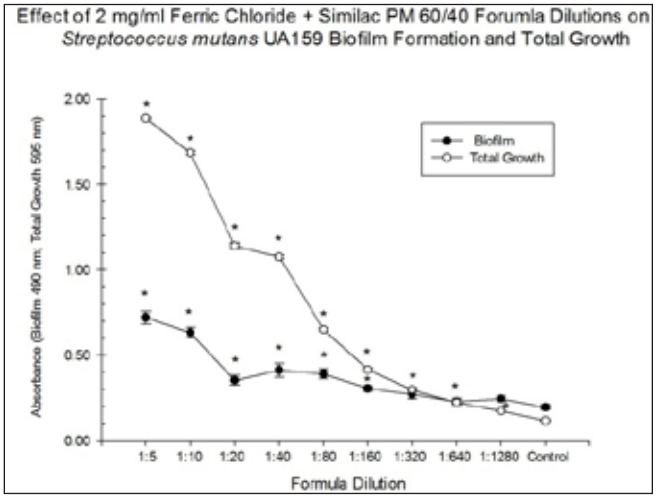
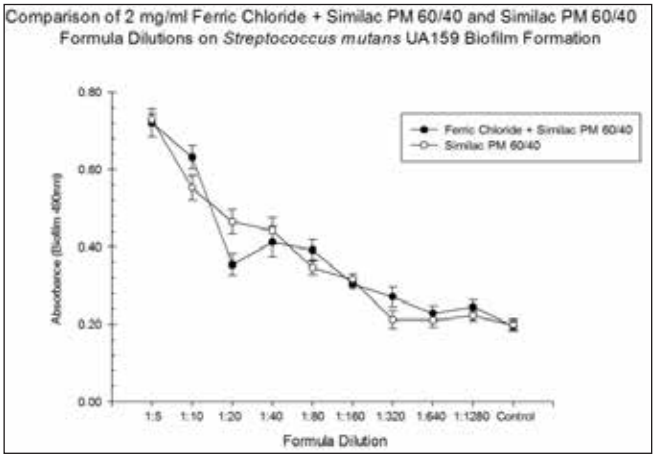


Figure 4. Effect of 2 mg/ml ferric chloride added to dilutions of the low iron Similac PM 60/40 infant formula on *S. mutans* biofilm formation and total growth compared to the TSB control without dextrose. Asterisks indicate statistically significant differences (p<0.05) compared to control values.





DISCUSSION

Sucrose-based formulas had more of a significant increase in biofilm growth than lactose-based formulas. Five sucrose-based formulas at higher concentrations (1:5, 1:20) had a significant increase in biofilm growth when compared to the six lactose-based formulas. Similac Sensitive RS (sucrose-based) had the most significant increase in biofilm growth at most dilutions compared to the other formulas. This is supported by an in vitro caries study by Prabhakar et al.[19] which concluded that the addition of an external carbohydrate source to milk, such as sucrose, increases the cariogenic potential and the extent of caries progression into dentin. In addition, a study by Bowen et al. [11] concluded that infant formulas had greater potential to promote cariogenic properties when compared to plain milk due to the higher carbohydrate variability.

Sucrose tested as an individual component had more of a significant increase on biofilm growth than lactose or iron when compared to the control. This finding is supported by a study completed by Campbell and Zinner,[16] who found that sucrose was the most cariogenic sugar tested and caused the most rapidly progressive carious process when compared to fructose, glucose, or lactose. However, high concentration of the individual components inhibited biofilm growth. The highest concentrations of sucrose, lactose, and ferric chloride acted as a preservative and inhibited bacterial growth.

There was no significant difference in biofilm growth when comparing the original Similac PM 60/40 to Similac PM 60/40 with added ferric chloride. Adding iron to a low iron content formula did not affect biofilm growth. The low iron formula had a significant increase in biofilm growth when compared to the normal iron formulas only at the highest concentration (1:5). Biofilm growth did not differ between low iron and high iron formulas, nor did it differ between normal and high iron formulas. The overall tendency of iron to not affect biofilm growth may be due to the small amount of iron included in the formulas.

CONCLUSION

The results of this study demonstrate that sucrose-based formulas cause an increase in *in vitro S. mutans* biofilm formation to a greater extent than lactose-based formulas. Sucrose as an individual component caused a significant increase in *S. mutans* biofilm formation compared to lactose and ferric chloride. The amount of iron in formula had a significant effect on biofilm formation only when comparing low iron formula to normal iron formula at the highest concentration (1:5). This information expands the current knowledge regarding the influence of different infant formulas and validates the necessity to begin an oral hygiene regimen once the first tooth erupts.

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